Synthetic Seed Technology in Ornamental Plants

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Keywords: cryopreservation, cut flowers, encapsulation, shrubs, synseed, trees

ABSTRACT

Synthetic seed technology is today an important tool, available to breeders and scientists, for the *in vitro* propagation and conservation of ornamental plants. After an introduction on the main procedures for synseed preparation, this chapter provides information on the protocols that have been developed for the encapsulation of various explants (zygotic and somatic embryos, axillary buds, nodal segments, protocorms and bulblets) from ornamental species (trees, shrubs and cut flowers). Original results about the germinability and the average time of germination from 7 ornamental species are also presented. The chapter ends with a review of papers dealing with the application of the synthetic seed technology to the slow growth storage and the cryopreservation of ornamental plant germplasm.

1. INTRODUCTION

Synthetic seed technology is one of the most important applications of plant tissue culture, as it combines the advantages of clonal propagation with those of seed propagation (i.e., storability, easy handling and transport, use of sowing equipment, protection against diseases and pests). In addition, the synthetic seeds are used today in advanced procedures of cryopreservation (such as the "encapsulation-dehydration" method) with very promising results for the long-term preservation of plant germplasm.

The first appearance of synthetic seeds (also called "artificial seeds" or "synseed") dates back to over 30 years ago and emerged from the idea of encapsulating a single somatic embryo inside an artificial seed coat, thus mimicking the natural seeds. Murashige (1977) was the first to produce an official definition of "synthetic seed": "an encapsulated single somatic embryo, i.e., a clonal product that could be handled and used as a real seed for transport, storage and sowing, and that, therefore, would eventually grow, either *in vivo* or *ex vitro*, into a plantlet". Although Murashige had clearly envisioned the potential of this new tool in terms of clonal propagation, he also commented: "but to be applicable, this cloning method must be extremely rapid, capable of generating several million plants daily and competitive economically with the seed method". Differently to zygotic embryos, which are protected by a seed coat and have an access to the nutrients that are accumulated in the cotyledons or in the endosperm, somatic embryos are naked and dependent on the culture medium. Hence, it was soon evident that the synseed had to be similar to the natural seed as much as possible, i.e., it required the development of appropriate procedures for the encapsulation and the accumulation of storage compounds, creating an "artificial endosperm".

As stated by Murashige's definition, synthetic seed technology was initially restricted to species in which somatic embryogenesis was possible. Later, Bapat and co-workers (1987) proposed to broaden the technology to the encapsulation of various *in vitro*-derived propagules, and they used axillary buds of *Morus indica* as a first example of this new application. The new concept paved the way for the encapsulation of explants other than somatic embryos, and to the formulation of a new definition of synseed (Aitken-Christie *et al*. 1995) as "artificially encapsulated somatic embryos, shoots, or other tissues which can be used for sowing under *in vitro* or *ex vitro* conditions". In other words, the "new" synthetic seed, lost the original bond of containing an embryo (zygotic or somatic), can now contain any kind of explant from tissue culture (such as axillary buds, shoot tips, nodal segments, bulblets, protocorms, callus samples, cells) which, following "germination", will evolve into a plantlet or a shoot ("synseed conversion") or will produce new cell proliferation ("synseed regrowth"). An extensive and well-documented review concerning the use of non-embryogenic explants for the production of synthetic seeds was produced by Standardi and Piccioni (1998).

Today, almost 30 years after Murashige's original definition, plenty of reports on synthetic seed are available in the literature, dealing with the encapsulation of somatic embryos from cereals (George and Eapen 1995), vegetables (Molle *et al*. 1993, Sanada *et al*. 1993, Ghosh and Sen 1994), fruits (Onay *et al*. 1996, Castillo *et al*. 1998), conifers (Roberts *et al*. 1993, Fowke *et al*. 1994) and aromatic grasses (Patnaik and Debata 1996). In addition, the number of reports concerning the encapsulation of non-embryogenic propagules has increased (e.g., Bapat *et al*. 1987, Mathur *et al*. 1989, Sharma *et al*. 1994, Pattnaik *et al*. 1995, Ballester *et al*. 1997, Sarkar and Naik 1998).

Here, after a brief description of the procedures for synseed preparation, we report on the recent advances in the application of the synthetic seed technology to ornamental plants (woody, shrub and cut-flower species).

Abbreviations: ABA, abscisic asid; **BA**, benzyladenine; **GA_3**, gibberellic acid; **IAA**, indole-3-acetic acid; **MS**, Murashige and Skoog medium; **NAA**, naphtalene acetic acid; **PLBs**, protocorm-like bodies

2. Synthetic seed preparation

2.1. Encapsulating (=gelling) agents

When looking at the history of synthetic seed technology, a wide number of encapsulating agents have been tested in time for their capacity to produce beads, such as agar, agarose, alginate, carboxymethyl cellulose, carragenan, ethylocellulose, gelrite, guargum, nitrocellulose, polyacrylamide, polyox and sodium pectate (Datta *et al*. 2001, Saiprasad 2001). For instance, Kitto and Janick (1985a) tested eight chemical compounds for the production of synthetic seed coats, among which a water-soluble resin, the polyethylene oxide homopolymer ("polyox"), was the best to coat embryogenic suspensions (i.e., cells, aggregates, callus clumps and embryos) of carrot. They prepared a 5% solution of polyox and made the synthetic seeds by mixing equal volumes of this solution and embryo suspensions. By dispensing 0.2 ml of the mixture onto Teflon sheets, a "detachable wafer" containing the embryos was obtained. When the original embryogenic suspensions were cultured on medium containing 1 µM ABA during the induction phase (14 days), the germination of the resin-coated embryos increased to 40%. Polyox was reported to have several positive characteristics as an encapsulating agent, i.e., (i) it formed a film after drying, (ii) it didn't support the growth of contaminants, and (iii) it was non-toxic for the encapsulated embryos. Later on, this methodology was used to obtain synthetic seeds of orange and celery as well (Kitto and Janick 1985b, Kim and Janick 1989).

Redenbaugh *et al*. (1988), after comparing several substances for making synthetic seed coats, proposed the use of a Na-alginate solution which could be turned into a hardened Ca-alginate gel by an ion-exchange reaction. Somatic embryos of alfalfa and celery were the first to be alginate-coated, reaching a synseed germination rate (=conversion to plantlets) which in alfalfa was over 85%. Since then, the alginate became by far the most used gelling agent for synthetic seed preparation. The main advantages of this compound are: the excellent water solubility and moderate viscosity of Na-alginate at room temperature, its easy availability at low cost, the long-term storability of the Na-alginate solution, the easy use of calcium salts for quick gellation and bead hardening at room temperature, the possibility to prepare synseeds of different hardness by changing the concentration of Na-alginate and/or the duration of the ion-exchange reaction, the absence of any kind of toxicity of the Ca-alginate matrix for explants, the possibility to mix the alginate with a nutritive medium to obtain an artificial endosperm. In addition, the rigidity of alginate beads provides an effective protection to the somatic embryos. However, one unfavourable characteristic of the alginate is the tacky nature of the capsules that are produced. Indeed, the alginate seed coats are very moist and they have a sticky surface, making the seeds adhere to each other and difficult to separate. Another limitation is due to the rapid dehydration of the alginate beads which, upon exposure to air, makes the synseeds become very hard in just a few hours, preventing or making the conversion of the enclosed explants into plantlets or shoots very difficult. Redenbaugh and co-workers (1987) were the first to understand the necessity of a hydrophobic layer at the surface of the synthetic seeds, avoiding bead adhesion and preventing water loss. Several chemical compounds have been tested in time for this purpose (see **2.2.**).

2.2. Methods of encapsulation

In addition to providing rigidity to the explant for the protection against physical damage, the synthetic seed coat should also permit the propagule to be converted into a plant or shoot (if a somatic embryo or an axillary bud), or to regrowth (if it consists of cells or of a callus sample). In other words, it should be able to deliver the nutrients, the growth regulators and the other components of the artificial endosperm which are necessary for the germination of the synseed, while at the same time to enable handling during storage, transportation and planting (Winkelmann *et al*. 2004). Among the various encapsulation methods which have been in time tested for the production of synthetic seeds, only the gelation method was found to be suitable to produce good (i.e., firm enough) capsules, and to support embryo survival (Redenbaugh *et al*. 1987).

In the most common encapsulation technique, an appropriate sterile solution (in general, 3%) of Na-alginate in pure water or, more often, in a nutritive medium (containing or not growth regulators) is mixed with the explants inside a small glass container, working under the sterile air of a laminar-flow hood. From this container, the explants are sucked with a pipette (whose tip have been trimmed to obtain a 2-4 mm hole) together with the Na-alginate solution (**Fig. 1A**). The Na-alginate is then released drop-by-drop (each drop containing a single explant; **Fig. 1B**) into a sterile solution of the complexing agent (a di-

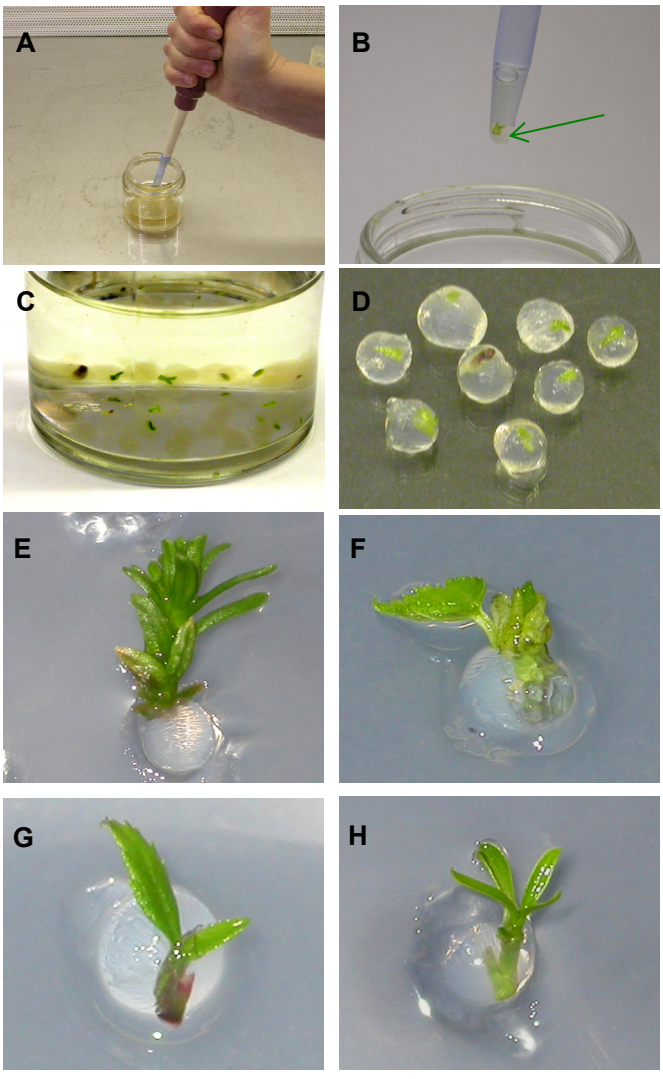

Fig. 1 A-D: Synthetic seed preparation. (**A**) the explants are sucked with a pipette, together with a quota of Na-alginate. (**B**) a drop (*arrow*) of Na-alginate, containing an explant, is going to be released in the complexing solution. (**C**) synseeds during the hardening in the $CaCl_2 \cdot 2H_2O$ solution. (**D**) synseeds after recovering and washing from the complexing solution. **E-H:** Examples of germination (=conversion to shoots) of ornamental plant synseeds (made with axillary buds or segmental nodes). In brackets is the time after the transfer of synseeds to the germination medium. (**E**) *Sequoia sempervirens* (27 days). (**F**) *Tilia cordata* (21 days). (**G**) *Photinia fraserii* (20 days). (**H**) *Nerium oleander* (21 days).

or tri-valent metal salt, such as calcium chloride or calcium nitrate). Alternatively, the explant is directly inserted into the forming drop before its detachment from the tip and its falling down into the complexing agent. In both techniques, an ion-exchange reaction occurs in 20-30 minutes, during which calcium replaces the sodium and the beads gel completely (**Fig. 1C**). After bead hardening, the synseeds are then collected with a sieve and washed with sterile water (**Fig. 1D**).

To overcome the problems related with high adhesion and the rapid desiccation of Ca-alginate beads, several hydrophobic coating agents have been tested over time, such as aluminium monostearate, Elvax 4260 (Dupont, SRI Int.), Gantrez ES, methylvinyl ether/maleic anhydride, gluteraldehyde, polylysine, and polyproline (Redenbaugh *et al*. 1988). Among them, Elvax 4260 showed a significant impediment to capsule drying. Khor *et al*. (1998) introduced the concept of a non-tacky, water-barrier coating of the alginate capsules, obtained by means of alginate-chitosan or alginate-gelatin encapsulation. In this technique, the beads consist of an inner alginate core, which simulates the endosperm of seeds, with either a chitosan or a gelatine coating, hard enough for the protection and easy handling of the synseeds.

Recently, other methods of explant encapsulation have been proposed, alternative to the conventional "drop-by-drop" release of alginate into the complexing calcium agent. A couple of them are worthy of mention, such as the "partially encapsulated synseed" (Mamiya and Sakamoto 2001). In this technique, after empty Ca-alginate beads (i.e., without explants) are prepared as described above, a hole is made in the capsules from which the explant (a somatic embryo) is then inserted to form the synseed. An advantage of this method is related to the fact that the embryo primordium is not embedded totally in the alginate matrix, thus preventing any possible physical inhibition to the embryo conversion to plantlet. However, the complexity of the technique, in comparison with the conventional "drop-by-drop" method, is a weak point which has prevented its diffusion. Another encapsulation procedure was developed with the aim of producing Ca-alginate hollow beads (Patel *et al*. 2000). Here, the explants (i.e., cells or shoot tips of potato and carrot) were suspended in a solution containing carboxymethylcellulose and calcium chloride, and then dripped into a Na-alginate solution. The technique allowed the explants to regrow (cells) or to be converted to shoots (shoot tips) directly inside the capsules. The advantage of this new concept is that, in contrast to the conventional method where the explant is often located near the surface, here the explant is positioned exactly at the centre of the bead, thus ensuring the complete protection of the embryo (or other explant) as in the natural seed.

In another procedure of synthetic seed production, which never achieved the popularity of the Na/Ca-alginate method, the explants are mixed in a temperature-dependent gel (such as Gelrite®), after which moulds of Gelrite (each mould containing one explant) are gelled as the temperature is lowered (Redenbaugh *et al*. 1987).

3. SYNTHETIC SEEDS OF ORNAMENTAL PLANTS

A consistent number of reports are today available, dealing with the application of synthetic seed technology to ornamental plants. The main species for which a complete procedure has been developed are listed in **Table 1** (shrubs and trees) and **Table 2** (cut flowers). The Tables give also details on the encapsulated explants (somatic or zygotic embryos, protocorms, axillary buds, bulblets, nodal segments) and on the germination rates of synseeds.

3.1. Synthetic seeds containing embryos, protocorms and bulblets

Most of the reports followed the conventional synthetic seed technology, with embryos (zygotic or somatic) completely embedded in a Ca-alginate matrix. The germinability of this kind of synseeds is sometimes lower than the control (natural seeds or naked somatic embryos). In *Camellia sinensis* (Seran *et al*. 2005) and *Cleopatra tangerine* (Nieves *et al*. 1998), for instance, germination rates of zygotic embryos encapsulated in beads of 3% alginate were lower than the ones of natural seeds. Similarly, synseeds made with somatic embryos of *Paulownia elongata* (Ipekci and Gozukirmizi 2003) and *Genista monosperma* (Ruffoni *et al*. 1994) showed a conversion to plantlets lower than the one of naked somatic embryos. On the other hand, synseeds of *Citrus reticulata* germinated at a percentage which was higher than the control (50% vs. 15%; Germanà *et al*. 1999).

When embryos are used for the production of ornamental plant synseeds, Na-alginate is generally used at concentrations of 2-3%. A lower concentration (1.5%) has been reported for the encapsulation of globular somatic embryos of *Cyclamen persicum* (Winkelmann *et al*. 2004). Similarly, the hardening of beads is generally performed in 50-100 mM of $CaCl_2 \cdot 2H_2O$, with exposure times ranging from 20 min (*Cyclamen persicum*; Winkelmann *et al*. 2004) to 60 min (*Pelongonium horturum*; Gill *et al*. 1994). Some papers report a marked influence on synseed "germinability" of both the alginate concentration of beads, and the concentration and the exposure time to the complexing agent. In *Paulownia elongata*, for instance, a fall in frequency of somatic embryo survival and conversion to plantlets was observed when the Na-alginate concentration of beads was reduced from 3% to 1%. Similarly, the highest germination rate was obtained when a 30-min exposure to 50 mM $CaCl_2 \cdot 2H_2O$ was applied for bead polymerization, in comparison with a 10-min exposure to 60 mM or 80 mM (Ipekci and Gozukirmizi 2003).

In Orchids, the most important cut flowers which are commercially propagated *in vitro*, the best conversion to plantlets (100%) of encapsulated PLBs of *Dendrobium* 'Sonia' was observed when 3% Na-alginate drops were hardened for 30 min in 75 mM $CaCl_2 \cdot 2H_2O$ (Saiprasad and Polisetty 2003). Differently, PLBs of *Geodorum densiflorum* required encapsulation in 4% Na-alginate and bead hardening in 50 mM $CaCl_2 \cdot 2H_2O$ (15-20 min) for maximum conversion to plantlets (88%; Datta *et al*. 2001). These results evidence that the concentration of both the components of encapsulation (the alginate and the calcium complexing agent) may require to be optimized when producing synthetic seeds of different Orchids species. Moreover, working with another *Orchidaceae* species, Khor *et al*. (1998) used chitosan or gelatine, together with Na-alginate, as water-barrier coating agents ("two-coat system"), in order to produce capsules containing seeds or protocorms of *Spathoglottis plicata*. Indeed, the mixture of alginate with chitosan or gelatine proved to be beneficial for the production of high-quality synthetic seeds, which germinated at a rate of up to 100%.

The inclusion in the bead of a medium, containing or not growth regulators, produces a sort of "artificial endosperm" which can be beneficial for synseed germination, overcoming the problems due to the lack of natural nutrients and endogenous hormones. The germinability of synthetic seeds of *Citrus reticulata* somatic embryos, for instance, was greatly improved by the presence of 1 μM GA_3 in the medium which was added to the alginate matrix (Germanà *et al*. 1999). Besides GA_3, also auxins (IAA, NAA) and cytokinins (zeatin, BA) are common components of the

artificial endosperm of ornamental plant synseeds. However, high rates of germinability are also reported for synseeds containing a hormone-free nutritive medium, as for *Paulownia elongata* (Ipekci and Gozukirmizi 2003), *Cyclamen persicum* (Winkelmann *et al*. 2004), *Genista monosperma* (Ruffoni *et al*. 1994), *Geodorum densiflorum* (Datta *et al*. 2001), *Lilium longiflorum* (Standardi *et al*. 1995) and *Pelongonium horturum* (Gill *et al*. 1994).

Table 1 Preparation and germination of synthetic seeds from explants of ornamental plants (shrubs and trees; Na-A, sodium alginate).

Species	Explants	Encapsulation[1]	Artificial Endosperm	Germination (%)[2]	Reference
Betula pendula	Nodal segments	2% (w/v) Na-A / 11 g/l $CaCl_2 \cdot 2H_2O$ (25-30 min)	None	100	Piccioni and Standardi 1995
Camellia japonica	Somatic embryos	3% (w/v) Na-A / 0.1 M $CaCl_2 \cdot 2H_2O$ (30 min)	MS + sucrose (3%) + GA_3 (14.4 µM) + IAA (28.5 µM)	63 (control, 65)	Janeiro *et al*. 1997
Camellia sinensis	Zygotic embryos	3% (w/v) Na-A / 100 mM $CaCl_2 \cdot 2H_2O$ (20 min)	None	42 (control, 73)	Seran *et al*. 2005
Citrus reticulata 'Avana'	Somatic embryos	2.5% (w/v) Na-A / 100 mM $CaCl_2 \cdot 2H_2O$ (30 min)	½ MS + malt extract (0.25 g/l) + sucrose (20 g/l) + galactose (4.5 g/l) ± GA_3 (1 µM)	(+ GA_3, 50; - GA_3, 26) (control, 15)	Germanà *et al*. 1999
Cleopatra tangerine	Zygotic embryos	3% (w/v) Na-A / 2% (w/v) $CaCl_2 \cdot 2H_2O$ (25 min)	MS + arginine (6 mg/l) + glutamic acid (8 mg/l) + GA_3 (1 µM)	93 (control, 100)	Nieves *et al*. 1998
Crataegus oxyacantha	Nodal segments	2% (w/v) Na-A / 11 g/l $CaCl_2 \cdot 2H_2O$ (25-30 min)	None	100	Piccioni and Standardi 1995
Morus spp.	Axillary buds	4% (w/v) Na-A/ 75 mM $CaCl_2 \cdot 2H_2O$	MS	100	Pattnaik *et al*. 1995
Morus spp.	Axillary buds	4% (w/v) Na-A / 75 mM $CaCl_2 \cdot 2H_2O$ (30 min)	MS + BA (4.4 µM)	*M. nigra* -*M. alba*, 95 (control, 70-89); *M. australis*, 98 (control, 0)	Pattnaik and Chand 2000
Paulownia elongata	Somatic embryos	3% (w/v) Na-A / 50 mM $CaCl_2 \cdot 2H_2O$ (30 min)	MS	53 (control, 66)	Ipekci and Gozukirmizi 2003
Syringa vulgaris 'M.me Lemoine'	Axillary buds	2% (w/v) Na-A / 50 mM $CaCl_2 \cdot 2H_2O$	½ MS + BA (5 mg/l) + NAA (0.01 mg/l)	15	Refouvelet *et al*. 1998

[1] In brackets is the time of bead hardening in the complexing agent.
[2] Synseed germination refers to the conversion of embryos and buds to plantlets and shoots, respectively. Control values refer to the germinability of non-encapsulated explants.

Table 2 Preparation and germination of synthetic seeds from explants of cut flowers (Na-A, sodium alginate; PLBs, protocorm-like bodies).

Species	Explants	Encapsulation[1]	Artificial Endosperm	Germination (%)[2]	Reference
Cyclamen persicum	Globular somatic embryos	Alginate beads :1.5% (w/v) Na-A / 68 mM $CaCl_2 \cdot 2H_2O$ (20 min) Hollow beads[3]: 1.5% carboxy-methylcellulose / 0.8% Na-A	½ MS	Alginate beads, 97 Hollow beads, 71 (control, 97)	Winkelmann *et al*. 2004
Dendrobium 'Sonia'	PLBs	3% (w/v) Na-A / 75 mM $CaCl_2 \cdot 2H_2O$ (30 min)	¾ MS + BA (0.44 µM) + NAA (0.54 µM)	100	Saiprasad and Polisetty 2003
Eustoma grandiflorum	Somatic embryos	2% (w/v) Na-A / 2% (w/v) $CaCl_2 \cdot 2H_2O$	MS + zeatin (0.5 mg/l)	22	Ruffoni *et al*. 1994
Genista monosperma	Somatic embryos	2% (w/v) Na-A / 2% (w/v) $CaCl_2 \cdot 2H_2O$	MS	29 (control, 50)	Ruffoni *et al*. 1994
Geodorum densiflorum	PLBs	4% (w/v) Na-A / 50 mM $CaCl_2 \cdot 2H_2O$ (15-20 min)	Knudson C	88	Datta *et al*. 2001
Lilium longiflorum	Bulblets	2.5% (w/v) Na-A / 11 g/l $CaCl_2 \cdot 2H_2O$ (25-30 min)	MS	95 (control, 92)	Standardi *et al*. 1995
Pelongonium horturum	Somatic embryos	3% (w/v) Na-A / 50 mM $CaCl_2 \cdot 2H_2O$ (60 min)	MS	88	Gill *et al*. 1994
Spathoglottis plicata	Seeds and protocorms	AC: 2.5% (w/v) Na-A / 0.15% (w/v) chitosan (20 min) AG: 2.5% (w/v) Na-A / 2% (w/v) gelatin (30 min)	AC: none AG: MS	Seeds: 100 (AC), 96 (AG); Protocorms: 100 (control, 100)	Khor *et al*. 1998

[1] In brackets is the time of bead hardening in the complexing agent.
[2] Synseed germination refers to the conversion of embryos, protocorms and bulblets to plantlets and shoots, respectively. Control values refer to the germinability of non-encapsulated explants.
[3] For encapsulation in hollow beads, somatic embryos were suspended in 1.5% carboxymethylcellulose solution and dropped into 0.8% Na-alginate under slow stirring. Alginate solution was diluted after 20 min, and totally discarded after 10 min.

3.2. Synthetic seeds containing axillary buds and nodal segments

In comparison with the embryos, the use of non-embryogenic explants for the preparation of synthetic seeds is much more recent (Standardi and Piccioni 1998). Notwithstanding, great advances have been made in recent years in synseed technology based on the use of axillary buds and nodal segments for the inclusion in alginate beads. In this system, germination refers to the conversion of the synseeds to shoots, i.e., to the bud break and the emergence of a shoot outside the alginate capsule. A complete plantlet is then obtained following a subsequent step of shoot rooting in an auxin-containing medium. Alternatively, the explants are pulse-treated with a root promoter before their encapsulation in alginate to achieve shoot formation and rooting at the same time, i.e., the direct conversion of the synseeds to plantlets (Welander and Pawlicki 1993, Capuano *et al*. 1998).

To date, effective protocols of synseed preparation have been developed for some important woody and shrub species (**Table 1**), using either axillary buds (*Morus* spp., Pattnaik and Chand 2000; *Syringa vulgaris*, Refouvelet *et al*. 1998), or nodal segments (*Betula pendula* and *Crataegus oxyacantha*, Piccioni and Standardi 1995). With the exception of lilac, very high conversion rates to shoots (95%-100%) were achieved with this kind of synthetic seeds.

At the IVALSA Institute of Florence (Trees and Timber Institute, in *http://www.ivalsa.cnr.it/*), belonging to the National Research Council (CNR) of Italy, the synthetic seed technology is developed for the application to cryopreservation procedures, such as "encapsulation-dehydration" and "encapsulation-vitrification" (see **4.**). These cryogenic procedures require synthetic seeds with characteristics of high germinability and a short average time of germination. **Table 3** shows some examples of conversion rates and average time of germination of synseeds from various ornamental species, among which are trees (*Sequoia sempervirens*, *Tilia cordata* and *Metrosideros excelsa*), shrubs (*Nerium oleander, Grevillea rosmarinifolia* and *Photinia fraserii*), and a succulent plant (*Kalanchoe*). All the synseeds were prepared by mixing axillary or apical buds with 3% Na-alginate in hormone-free MS medium, which was then released drop-by-drop (each drop containing one explant) in 100 mM $CaCl_2.2H_2O$. After bead hardening (30 min), the synseeds were plated on gelled hormone-free MS medium, and stored at 4°C in the dark. For germination, the synseeds were transferred to standard culture conditions, i.e., at 23 ± 1°C under a 16h photoperiod at 60 µmol m^{-2} s^{-1} photosynthetically active radiation provided by cool-white fluorescent lamps. Although the conversion rates were very variable (from a minimum of 36% for synseeds of *Kalanchoe* up to 96% for *Nerium oleander*), it is interesting to note that, on average, all the synthetic seeds germinated in a similar lapse of time, i.e., during the third week after their transfer to standard culture conditions (**Figs. 1 E-H**).

Table 3 Germination rates and average time of germination of synthetic seeds containing axillary buds from various ornamental plants (trees, shrubs and succulents). As for synseed preparation and germination conditions, see text (*unpublished data*).

Species	Germination (%)[1]	Average time of germination (days)[2]
Trees		
Sequoia sempervirens	70.0	17.7
Tilia cordata	88.0	17.8
Metrosideros excelsa	43.9	13.0
Shrubs		
Nerium oleander	96.7	18.5
Photinia fraserii	57.6	17.3
Grevillea rosmarinifolia	50.0	13.0
Succulents		
Kalanchoe	36.4	19.0

[1]Germination refers to the conversion of synthetic seeds to shoots

[2] Average time of germination (days) calculated as follows: $\Sigma (N_xT_x) /$ n° of germinated synseeds, where N_x = n° of synseeds germinating within consecutive intervals of time, T_x = n° of days between the beginning of the test and the end of the specific interval of time. Control values refer to the germinability of non-encapsulated explants.

4. APPLICATION OF THE SYNTHETIC SEED TECHNOLOGY TO THE PROPAGATION AND THE CONSERVATION OF ORNAMENTAL PLANTS

Many important ornamental plants either do not produce viable seeds (thus being propagated only by cutting or grafting), or even if they can be propagated by seeds, several problems are faced, including inbreeding depression and non-homogeneity of cultivars. To avoid that, controlled breedings are often necessary, a hand labour practice which increases the price of the seeds. One example is the production of Cyclamens (Winkelmann *et al*. 2004). In addition, in Orchids, the very small seed size and the requirement of an associatation with mycorrhizal fungi are great limitations to seed propagation. In addition, traditional *in vivo* propagation methods are time consuming and very costly (Saiprasad and Polisetty 2003).

For all the above reasons, the development of *in vitro* propagation methods, such as micropropagation and somatic embryogenesis, has represented a milestone for the reproduction of ornamental plants, providing a powerful tool for mass propagation, as well as for the production of transgenic plants with altered colour and/or scent. In spite of that, in Europe, only a small proportion of the annual production of some ornamental species (i.e., Lilac) are currently propagated *in vitro*, mainly due to the low proliferation rates affecting several species or varieties, as well as to problems faced with the acclimation of plants (Refouvelet *et al*. 1998). However, in the near future, the synthetic seed technology could be the key to overcome these problems, e.g., through automated procedures of synseed production (Aitken-Christie *et al*. 1995) followed by autotrophic micropropagation (Jeong *et al*. 1995). In fact, as the concept of synthetic seed involves the use of small propagules and enables the direct sowing of this material *in vitro* or *in vivo*, the technology could provide great flexibility to the breeders, not only reducing the costs when large quantities of propagules are required for handling, shipping and planting, but also eliminating the acclimation step when direct sowing *in vivo* is applied (Onishi *et al*. 1994).

Another potential application is the use of the synseeds as a carrier for micro-organisms, plant growth regulators, pesticides, fungicides, nutrients and antibiotics (Saiprasad 2001). For instance, in the case of Orchids, the seeds need to be infected by symbiotic mycorrhizal fungi to increase plant access to soil resources (Arditti *et al*. 1984), resulting also in an enhanced tolerance to biotic and abiotic stresses (Clement 1985, Rasmussen 1995). A method of encapsulation of orchid seeds in alginate-chitosan or alginate-gelatin beads, together with the mycorrhizal fungus *Rhizoctonia*, has already been proposed (Tan *et al*. 1998), achieving an infection rate of over 80%.

One important recent application of the synthetic seed technology concerns the preservation of plant germplasm, including endangered species, wild forms, ancient and obsolete varieties. The concept of germplasm conservation *in vitro* involves both the slow growth storage of shoot cultures (mainly at few degrees above 0°C), and the long-term preservation of explants (shoot tips, nodal segments, zygotic and somatic embryos, cells and callus samples) in liquid nitrogen, at -196°C (Lambardi and De Carlo 2003). The cryogenic technology, in particular, received recently a great improvement by the use of synthetic seeds with the development of procedures named "encapsulation-dehydration" and "encapsulation-vitrification" (Panis and Lambardi 2005).

Slow growth storage provides a useful alternative for the medium-term preservation of ornamental species (**Table 4**), i.e., with conservation times up to 10 months for somatic embryo synseeds of *Cyclamen persicum* (Ruffoni *et al*. 2002). In Orchids, encapsulated PLBs of *Oncidium* and *Dendrobium* species could be preserved at 4°C for 45 and 60 days, respectively, maintaining maximum germination rates (100%; Saiprasad and Polisetty 2003). However, in both species, a decrease in synseed conversion to plantlets was observed when the storage at low temperature was prolonged (not shown). In contrast, PLBs synseeds of *Geodorum densiflorum* could be stored for up to 120 days with a germination rate of

Table 4 Germination rates of synthetic seeds of ornamental plants after storage at low temperature (PLBs, protocorm-like bodies).

Species	Explants	Storage conditions	Germination (%)[1]	Reference
Camellia japonica	Somatic embryos	4°C, 60 days	30 (control, 22)	Janeiro *et al*. 1997
Citrus reticulata 'Avana '	Somatic embryos	4°C, 30 days	25 (control, 5)	Germanà *et al*. 1999
Cyclamen persicum	Somatic embryos	7°C, 300 days	60	Ruffoni *et al*. 2002
Cyclamen persicum	Globular somatic embryos	4°C, 30 days	alginate beads, 68; hollow beads, 20 (control, 68)	Winkelmann *et al*. 2004
Dendrobium 'Sonia'	PLBs	4°C, 60 days	100	Saiprasad and Polisetty 2003
Geodorum densiflorum	PLBs	4°C, 120 days	86 (control, 0)	Datta *et al*. 2001
Jakaranda mimosaefolia	Shoot tips	20°C, 180 days	70 (control, 70)	Maruyama *et al*. 1997
Lilium longiflorum	Bulblets	4°C, 30 days	95 (control, 91)	Standardi *et al*. 1995
Morus spp.	Axillary buds	4°C, 60 days	100	Pattnaik and Chand 2000
Morus spp.	Axillary buds	4°C, 90 days	18 (control, 0)	Pattnaik and Chand 2000
Oncidium 'Gower Ramsay'	PLBs	4°C, 45 days	100	Saiprasad and Polisetty 2003
Paulownia elongata	Somatic embryos	4°C, 60 days	32 (control, 32)	Ipekci and Gozukirmizi 2003
Pelongonium horturum	Somatic embryos	4°C, 45 days	70	Gill *et al*. 1994
Rosa hybrida 'King's Ransom'	Somatic embryos	4°C, 40 days	30	Jayasree and Devi 1997
Syringa vulgaris	Axillary buds	5°C, 50 days	87-75; (control, 0-63)[2]	Refouvelet *et al*. 1998

[1] Synseed germination refers to the conversion of explants to plantlets or shoots. Control values refer to the germinability of non-encapsulated explants.

[2] First rates: cv Madame Lemoine; second rates: cv Contesse d' Harcourt.

86%; it is noteworthy that, after the same period, the recovery of non-encapsulated PLBs was nil (Datta *et al*. 2001). In comparison with the control, a beneficial effect due to the slow growth storage (from 3 to 9 months) of synthetic seeds, is also reported for somatic embryos of *Camellia japonica* (Janeiro *et al*. 1997), *Citrus reticulata* (Germanà *et al*. 1999) and *Paulownia elongata* (Ipekci and Gozukirmizi 2003), axillary buds of *Morus* spp (Pattnaik and Chand 2000) and *Syringa vulgaris* (Refouvelet *et al*. 1998), as well as for bulblets of *Lilium longiflorum* (Standardi *et al*. 1995).

Although slow growth storage technique is widely used by micropropagation laboratories for the short- and medium-term conservation of stock cultures, germplasm stored this way is vulnerable to losses due to equipment failure, microbial contamination at subculture time and the effects of somaclonal variation. On the contrary, cryopreservation provides a feasible option for the long-term preservation of explants, once effective procedures of ultra-rapid freezing are optimized and explant regrowth is ensured after thawing and plating. At a cryogenic temperature, the rate of chemical and biophysical reactions is practically nil, so that the growth of the frozen organ/tissue is hampered. Hence, in theory, germplasm cryopreservation could be considered unlimited in terms of time. Since its first presentation by Fabre and Dereuddre (1990), the cryopreservation method based on alginate encapsulation of explants and bead dehydration (named "encapsulation-dehydration") has been developed for a wide range of plant species (Panis and Lambardi 2005). In this method, explants (usually shoot tips or embryos) are firstly encapsulated in alginate synseeds, which are then treated with a high sucrose concentration, dried down to a moisture content of 20-30% (under air flow or using silica gel) and subsequently rapidly frozen in liquid nitrogen. Although the procedure can be considered rather lengthy and labour-intensive, it has been observed that the presence of a nutritive matrix (the bead) surrounding the explant can promote its regrowth after thawing. Recently, a new technique has been proposed, named "encapsulation-vitrification" (Sakai 2000). The procedure combines the encapsulation of explants with the application of a vitrification mixture, generally the Plant Vitrification Solution n°2 (PVS2, by Sakai *et al*. 1990).

Although the application of the "encapsulation-dehydration" and the "encapsulation-vitrification" techniques to the cryopreservation of ornamental species is still recent, very promising results have been already achieved (**Table 5**). Indeed, following their preservation at -196°C, the germination rates of synseeds (containing shoot tips) ranged from 25% in *Rosa multiflora* (Lynch *et al*. 1996) up to 85% in *Chrysanthemum morifolium* (Sakai *et al*. 2000). Similarly, encapsulated somatic embryos of *Citrus* spp. (Gonzalez-Arnao *et al*. 2003) and *Iris nigricans* (Shibli 2000), as well as embryonic axes of *Citrus madurensis* (Cho *et al*. 2002), survived satisfactorily to cryopreservation, with recovery rates up to 86%. The dehydration times of synthetic seeds, required to enhance explant tolerance to ultra-rapid freezing, generally ranges from 2-3 hours when in silica gel, and from 4-6 hours when under the sterile air of a laminar flow hood. Finally, it should be noted that also the "encapsulation-vitrification" technique, although at present only reported for *Gentiana* spp. (Tanaka *et al*. 2004) and *Prunus domestica* (De Carlo *et al*. 2000), seems very promising.

Table 5 Germination rates of synthetic seeds of ornamental plants after cryopreservation by "encapsulation-dehydration" or "encapsulation-vitrification" and direct immersion in liquid nitrogen (SG, silica gel; AF, laminar air flow; PVS2, Plant Vitrification Solution n°2).

Species	Explants	Dehydration/Vitrification	Germination (%) [1]	Reference
Auricula	Shoot tips	Dehydration: SG, 23 h	77	Hornung *et al*. 2001
Chrysanthemum morifolium	Shoot tips	Dehydration: SG, 3 h	85	Sakai *et al*. 2000
Citrus madurensis	Embryonic axes	Dehydration: SG, 3 h	65	Cho *et al*. 2002
Citrus spp.	Somatic embryos	Dehydration: AF, 5 h	86	Gonzàlez-Arnao *et al*. 2003
Dianthus caryophyllus	Shoot tips	Dehydration: AF, 4 h	48	Halmagyi and Lambardi 2006
Gentiana spp.	Shoot tips	Vitrification: PVS2, 60-140 min	74	Tanaka *et al*. 2004
Iris nigricans	Somatic embryos	Dehydration: AF, 4-6 h	46	Shibli 2000
Prunus domestica L.	Shoot tips	Vitrification: PVS2, 3 h	47	De Carlo *et al*. 2000
Rosa multiflora	Shoot tips	Dehydration: SG, 2 h	25	Lynch *et al*. 1996

[1] Synseed germination refers to the conversion of explants to plantlets or shoots after cryopreservation.

5. CONCLUSIONS

Although of more recent development if compared to other categories of plants, the synthetic seed technology is today an important tool, available to breeders and scientists, for the propagation *in vitro* of ornamental species, including trees, shrubs and cut flowers. Protocols of encapsulation have been already optimized for various explants (zygotic and somatic embryos, axillary buds, nodal segments, protocorms and bulblets) from numerous ornamental species, achieving high synseed conversion rates, relatively short germination times and the production of high-quality plantlets. In addition, it must be underlined that the synthetic seed technology has greatly contributed to a further improvement of cryopreservation procedures. This biotechnologically advanced approach to the *ex-situ* plant conservation has to be considered of strategic importance for the long-term preservation of endangered and valuable germplasm from economically important ornamental species.

ACKNOWLEDGEMENTS

M Lambardi and C Benelli research activity is financially supported by the National Research Council (CNR) of Italy, Department "Agroalimentare", Project "Risorse biologiche e tutela dell' agroecosistema". Thanks to Sara Pignattelli for her technical assistance, to Prof. F Gumusel, Director of the Department of Biology of the G.I.T. of Kocaeli (Turkey), and to Prof. A Akcin for their continuous support to the scientific activity of EA Ozudogru and Y Ozden-Tokatli. A special thanks to the "Azienda Agricola Vivai Battistini" of Cesena (Italy) for providing a grant to EA Ozudogru and Y Ozden-Tokatli.

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